Flat agar and optimized media enable high-throughput *C. elegans* imaging in 96-well plates

Imaging *C. elegans* in 96-well plates enables large-scale screens, but raising worms in small wells isn't straightforward. We developed and optimized a broadly applicable protocol that produces flat agar surfaces for automated culture and microscopy of small animals.

Published Aug 10, 2026

Arcadia Science

DOI: 10.57844/arcadia-h01v-2nxt

Purpose

High-throughput imaging of *Caenorhabditis elegans* enables large-scale phenotypic screens of genetic mutations, drugs, and other perturbations — experiments that would be intractable to perform on 60 mm plates or slides. Many existing devices for high-throughput *C. elegans* imaging are incompatible with multichannel pipettes and liquid handlers, time-consuming to prepare, or susceptible to desiccation during long-term studies. A simpler alternative is to pour agar directly into 96-well plates. Unfortunately, agar forms a meniscus in the well, creating a curved surface that requires z-stack acquisition to capture all animals.

We tested several ways to flatten the agar surface, including a mechanical press that we termed the “MeniSquish” and found that centrifuging agar in 8-well strips eliminated the meniscus and gave minimal tilt in the agar. This approach consistently yields full plates of flat wells suitable for single-plane imaging, using only equipment found in most labs and a 3D-printable accessory. During our work, we compiled several other ways to improve imaging conditions, such as reducing the concentration of phosphate and peptone in the media.

Although we developed this for *C. elegans*, the protocol should benefit anyone imaging agar-dwelling organisms in high-throughput formats.

The strategy

As part of our efforts to model disease in *C. elegans*, we sought a simple, scalable way to track basic organismal features, including growth rate, size, morphology, and fecundity, throughout the life cycle. Our goal was to deploy this approach across diverse mutational backgrounds, RNAi libraries, and chemical and drug treatments to rapidly screen for macroscopic phenotypes.

Many high-throughput *C. elegans* imaging solutions exist, including the WorMotel [1], the Worm Corral [2], and the Terasaki tray approach [3]. Each of these approaches has drawbacks: Non-96-well formats can be incompatible with multichannel pipettes and liquid handlers, chemical worm deterrents introduce experimental confounds, and some setups are time-consuming to prepare or prone to desiccation during long-term studies.

Here, our goal was to develop the simplest possible 96-well setup, in which multichannel pipettes and liquid handlers can automate seeding of OP50 (the standard *E. coli* food strain) and *C. elegans* deposition, and automated microscopes can image whole plates with minimal user input.

The problem

Though pouring agar into 96-well plates seems like the simplest strategy, it creates a meniscus in each well — surface tension draws the liquid up the walls as it sets, leaving a curved rather than flat surface. Worms crawl along this curve, so capturing every animal requires a z-stack acquisition of approximately 450 µm, which multiplies imaging time and inflates data volume (Figure 1). Worse, worms keep moving during stack acquisition, rendering projections uninterpretable and undermining downstream segmentation.

Our solution

We developed a simple casting workflow that uses a standard plate centrifuge to flatten agar in 8-well strips, which then snap into a 96-well frame (Figure 1).

Because any number of strips can be included, the same workflow scales from a handful of wells for a pilot to a full plate for a large screen. The assembled plates are directly compatible with standard 96-well tooling for automated seeding and imaging. Single-plane acquisition of every animal cuts imaging time, eliminates motion artifacts from stack projection, and reduces data volume. The agar volume and standard 96-well lid limit desiccation, supporting long-term studies.

We share this protocol openly for anyone working with agar-dwelling organisms in high-throughput formats.

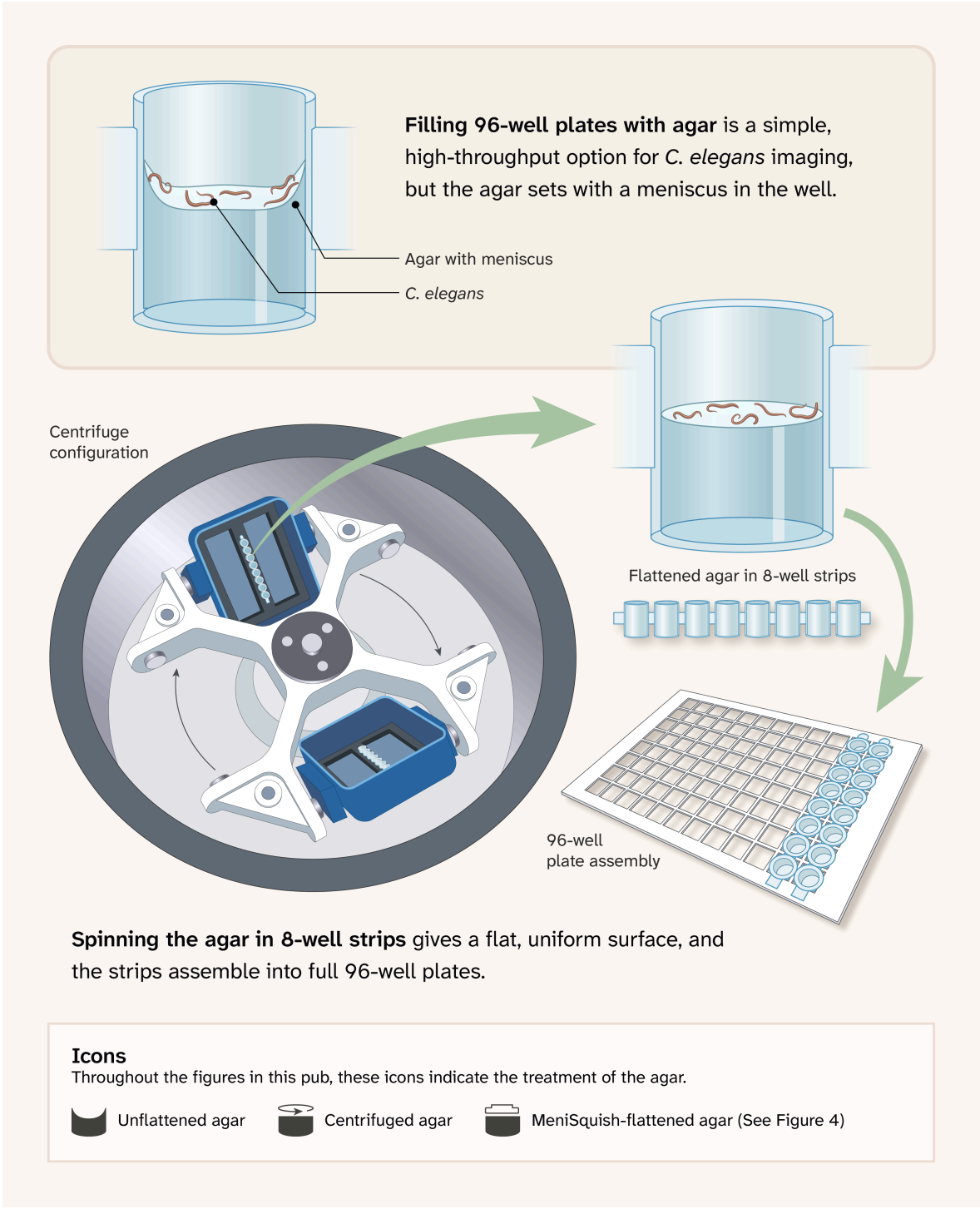

Figure 1. **Centrifuging agar in 8-well strips creates a flat surface that places all worms in one focal plane.**

Agar poured into wells sets with a meniscus, leaving worms at different focal planes and complicating imaging. Centrifuging strip wells, centered in the bucket with a special holder, flattens the agar so all worms sit in a single focal plane. We then snap the strips into a 96-well frame, using as many as the experiment requires. The icons at the bottom denote the three agar treatments we compare throughout the pub: unflattened, centrifuged, and mechanically flattened with the MeniSquish (Figure 4).

The resource

The following subsections describe how we used centrifugation to generate consistently flat agar surfaces in a 96-well format for high-throughput *C. elegans* imaging, as well as several imaging optimizations we explored.

For details on how to use our 8-well strip approach to generate level agar, jump to "Preparing plates with level agar."

Leveling the agar

We tried several approaches to flatten the agar in 96-well plates (see "Things that didn't work") and ultimately found centrifugation to be most effective. Our centrifuge buckets hold 96-well plates such that a line through a row (e.g., A1–A12) is perpendicular to the ground while spinning, and thus the very center, between rows D and E, best aligns with the centrifuge's axis of rotation. When we filled all the wells with agar and centrifuged them at 1,000 × g for 10 min while the agar set, the central two rows (D and E) came out nearly flat, with a slight tilt reflecting each well's displacement from the rotor's circular path. Rows farther from the center showed progressively steeper tilts, making them unusable.

To minimize plate waste and spin only the number of wells each experiment needed, we switched to 8-well strip wells. We made a custom 3D-printed strip-well centrifuge holder that centers the 8-well strips on the centrifuge's axis of rotation, making all wells uniformly flat with minimal tilt. After centrifugation, we snapped the flattened strips into a 96-well frame at the positions and scale required for the experiment. The assembled plates have a flattened agar surface in every well with only a slight residual tilt, and they work directly with standard 96-well tooling for automated OP50 seeding, worm deposition, and imaging.

To try this approach yourself, check out the more detailed methodology in "Preparing plates with level agar" and download the files to 3D-print the centrifuge adapter below. If you have any trouble applying our approach, don't hesitate to post a question on this pub.

Download **3D-printing files** for our custom 8-well strip

centrifuge holder from NIH 3D.

Bringing the worms into focus

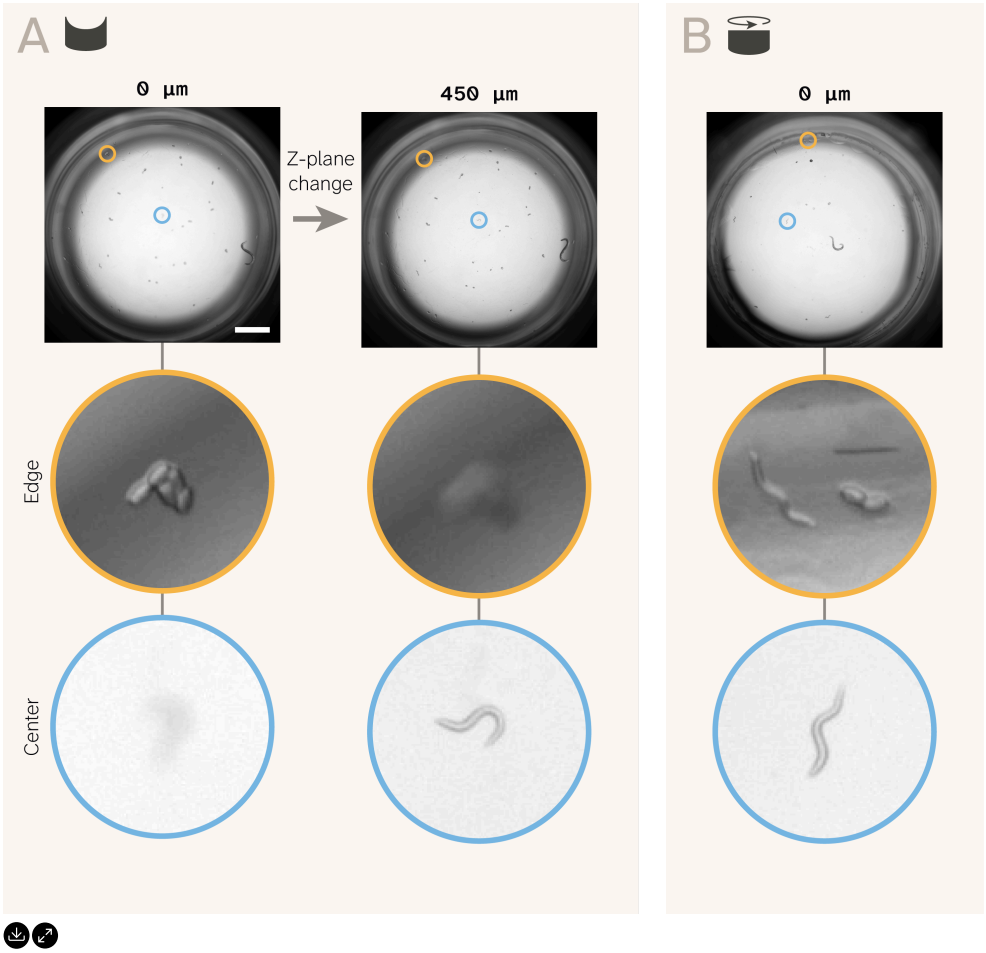

Figure 2. **Centrifuging agar allows single-plane *C. elegans* imaging.**

(A) In unflattened NGM wells, objects across the well span a z-range of 0–450 µm. The cluster of eggs at the edge (orange) is in focus at 0 µm, whereas the L1 larva at the center (blue) comes into focus only at 450 µm. Scale bar, 1 mm.

(B) In centrifuged NGM wells, the whole well images in a single plane. Eggs and an L1 larva at the edge (orange) are in the same focal plane as an L1 larva at the center (blue).

To assess the value of centrifugation, we pipetted nematode growth medium (NGM) into 8-well strips and either allowed it to set naturally or centrifuged it. We placed the strips in the 96-well frame, seeded the wells with bacteria, added bleach-synchronized L1 *C. elegans,* and imaged over three days of development. We created Opentrons protocols for each of these steps (bleach synchronization, bacterial seeding, and worm plating).

While the Opentrons worm plating program successfully automates animal delivery, it's imprecise, and the number of worms delivered can vary significantly, from zero to tens of animals, depending on how many animals went into the bleach preparation. We hope to develop an affordable system that delivers an exact number of worms to each well, and would appreciate any suggestions on currently available approaches. For the experiments described next, we pipetted L1s by hand from our bleach preparation, aiming for ~1–3 animals per well.

We imaged the animals immediately after plating and on the following three days. We examined the focal range for each condition by capturing 11 z-planes at 150 µm spacing. Naturally set plates required four slices covering 450 µm to capture all focal planes (Figure 2, A), whereas we only needed one slice from the centrifuged wells for analysis (Figure 2, B). Because the exact in-focus plane varies slightly from well to well — likely due to variation in agar loading and desiccation over time — we still captured multiple z-planes per well and wrote a custom script (see "Imaging and analysis") to automatically select the correct in-focus plane for each one. Overall, a larger proportion of the plate is captured in a single frame of centrifuged plates than naturally set ones, as the edges are no longer out of focus.

> **Code**, including our Opentrons protocols and in-focus plane slicer, is available in this GitHub repo (DOI: 10.5281/zenodo.21878776).

Cleaning up the picture with optimized media and dyes

Early in this work, we noticed salt crystals forming on the NGM surface over time, an issue we have also observed on standard 60 mm plates. Though it doesn't appear to affect the animals, the crystals interfered with image segmentation (Figure 3, A). To address this, we looked for salts we could reduce in the NGM recipe. The standard WormBook NGM recipe [4] calls for 25 mM phosphate buffer. However, some labs use 12.5 mM without affecting the animals [5]. We found that the lower-phosphate recipe eliminated crystal formation and adopted it for all

experiments. We haven't noticed any effects on worm growth or phenotypes, but haven't tested this explicitly.

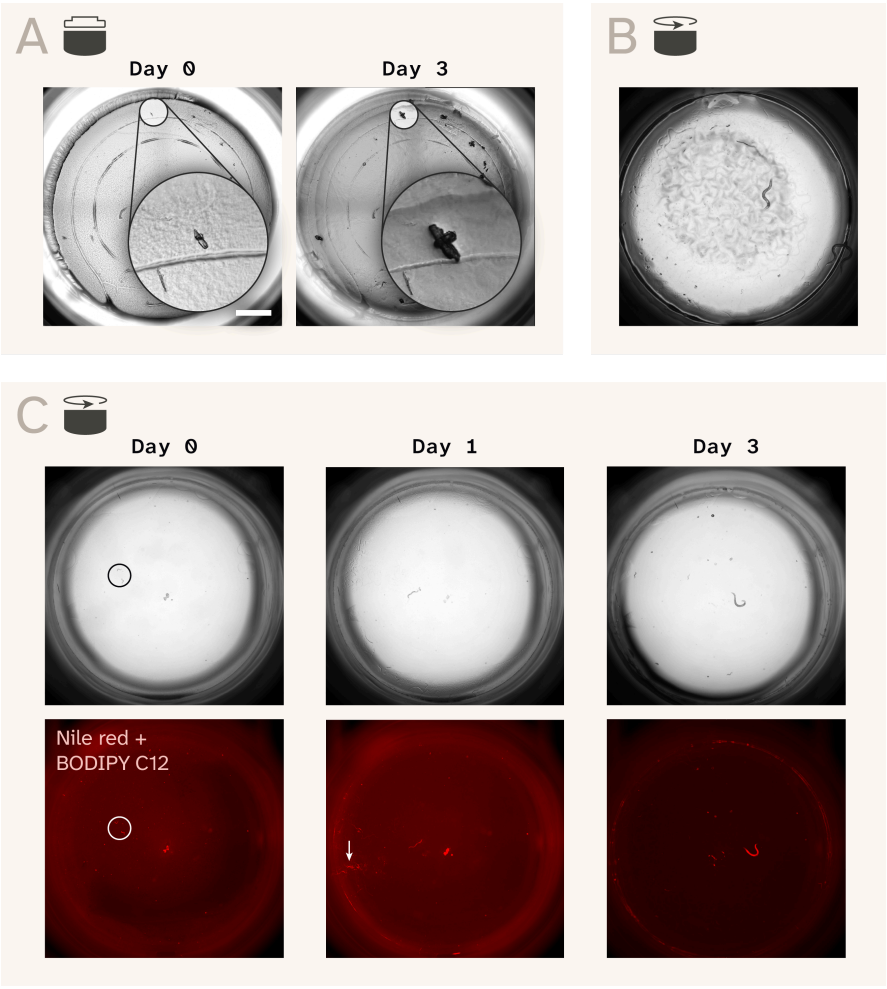

Figure 3. **Optimized conditions for 96-well plates.**

(A) An NGM well with small crystals growing (enlarged in inset) from day 0 (left) to day 3 (right). We flattened these wells with the MeniSquish. Scale bar, 1 mm.

(B) Worms disturb a thick mat of *E. coli* on full-peptone NGM, creating patterns that complicate segmentation. We flattened these wells via centrifugation.

(C) Top: Brightfield images of animal development across three days, beginning at starved L1 in centrifuged plates with low-peptone NGM. Bottom: Paired red fluorescent images of animals labeled with Nile red and BODIPY C12. Circles mark an L1 larva in both channels. The arrow indicates non-specific background. We flattened these wells via centrifugation.

Another issue that complicates segmentation is the thick bacterial mat that the OP50 *E. coli* forms over time. Worm movement through the mat disturbs it, producing complex patterns that are often classified as worms during processing (Figure 3, B). Other labs have addressed this problem by using low-peptone NGM, which produces a thinner lawn [6] [7]. In our experiments, this significantly reduced

the thickness of the *E. coli* mat, thereby facilitating worm identification. On low-peptone NGM, 1 µL of OP50 is sufficient for at least three worms (the most we plated in a well) to reach adulthood and lay eggs within a normal three-day period, though we haven't tested for greater effects on animal physiology (Figure 3, compare B with C).

Even with these optimizations, brightfield segmentation can be unreliable for small or sparsely pigmented animals. To improve detection, we turned to adding fluorescent dyes to the OP50 before seeding. We tried Nile red and BODIPY 558/568 C12, both separately and mixed. We selected these dyes based on their established use in *C. elegans* and because the lipids they target are broadly distributed throughout the animals and deposited in the eggs [8] [9] [10]. When supplied to the wells, these dyes successfully stained the worms, with Nile red especially strong in L1 larvae immediately upon deposition onto the food (Figure 3, C, circle). However, while the dyes labeled the worms, they still didn't stand out strongly against the background, and competed with autofluorescent particles on the plate. We also noticed the disturbed OP50 fluoresced strongly, creating worm-like patterns that were difficult for segmentation to handle. Finally, neither dye accumulated significantly in the eggs. We're still exploring other dyes that we might deliver with the OP50 to aid animal segmentation. If you have suggestions for dyes or other ways to segment worms, we'd love your feedback.

Overall, our validation experiments showed we could create a flat agar surface and maximize the imageable area in each well in a single focal plane with a system that uses only standard or 3D-printed lab equipment and remains compatible with 96-well automation tools, enabling high-throughput *C. elegans* imaging.

Things that didn't work

Before choosing centrifugation, we tried several mechanical approaches to flatten the agar surface. One was to pour a flat bed of agar into a single-well plate and press a 96-well-shaped insert into the set agar, pushing the walls into an already flat surface; this produced tears and deformations as the walls cut through. We next tried 3D-printing the lower portion of a 96-well plate, filling the wells with agar to the top so no surface existed for a meniscus to form, letting the agar set,

and then continuing to print the upper walls on top — but heat from the printer nozzle desiccated the agar, causing it to peel away.

We then designed a tool to squish the meniscus, the "MeniSquish," with pegs sized to descend 3 mm into each well, leaving enough clearance around the perimeter for excess agar to escape as the pegs pressed down (Figure 4). This produced uniformly flat surfaces, but the displaced agar collected in a ring around each well's inner wall, providing an escape route for worms, which occasionally fell back into the well (see the box below for more detail). In general, we found that any physical contact with the agar disrupted its smoothness and caused it to separate from the walls, allowing moisture to accumulate in the gap and worms to leave the surface. For our protocols, we wanted smooth agar bound to the walls, so we focused on flattening approaches that required no contact with the agar. However, as the MeniSquish could be useful for other organisms or for additional troubleshooting with *C. elegans*, we include a description and the CAD file so you can print your own version.

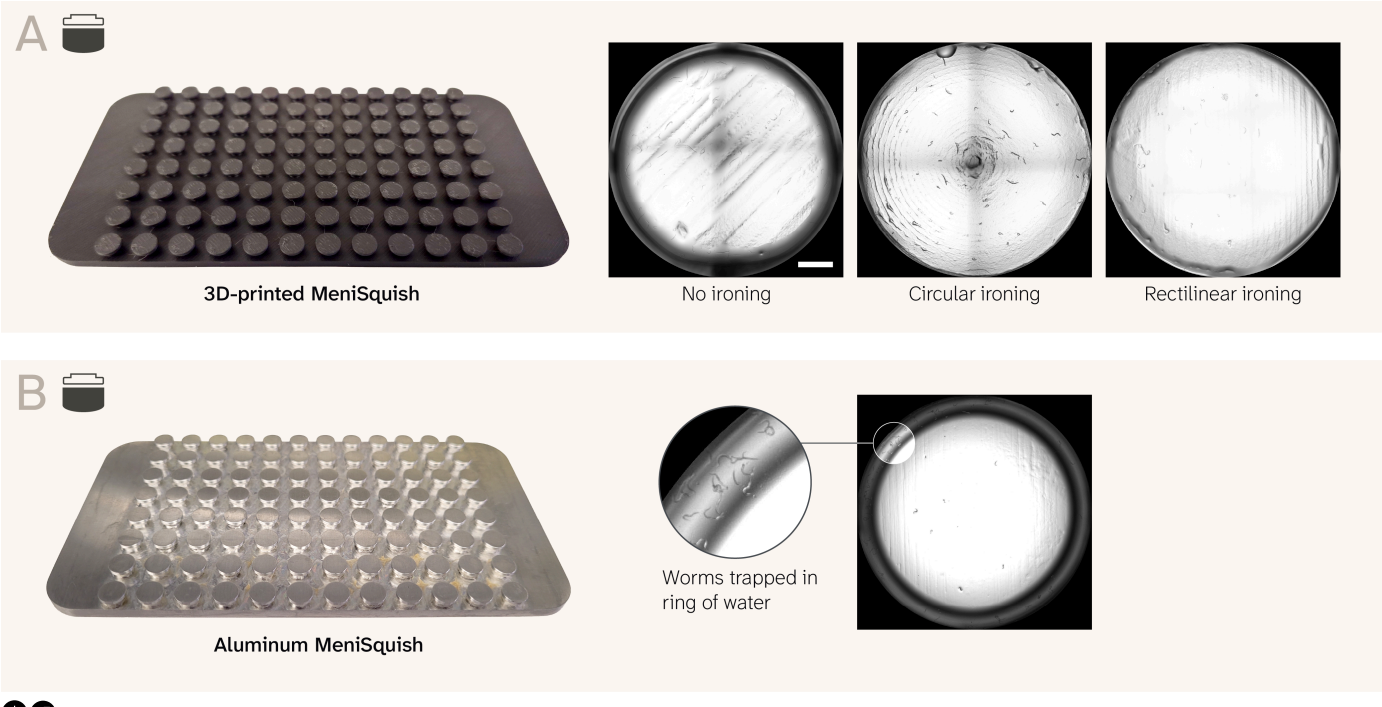

Figure 4. **The "MeniSquish" agar-flattening tool.**
(A) A 3D-printed MeniSquish and images of flattened agar in wells. The left image is from a MeniSquish printed without ironing, while the center and right images are from MeniSquishes printed with circular and rectilinear ironing, respectively. Scale bar, 1 mm.
(B) An aluminum MeniSquish and an image of the flattened agar. The inset shows worms trapped in a ring of water at the well edge.

▶ **Attempts at optimizing the MeniSquish**

Download **3D-printing files** for our custom **MeniSquish tools** from NIH 3D: MeniSquish, MeniSquish scraper.

Materials and methods

Preparing the worms

We maintained N2 worms at 20 °C on 60 mm plates with standard NGM (US Biological, N1000). For imaging experiments, we collected gravid worms by washing plates with 1 mL of water, then transferred them into 1.1 mL deep-well 96-well plates (Axygen, P-DW-11-C-S). We performed bleach synchronization by alkaline hypochlorite treatment on an Opentrons Flex liquid handler fitted with a 96-channel 1,000 µL pipette. Briefly, we washed worms once with water, treated them with bleaching solution (17 parts water to two parts bleach to one part 5 M KOH by volume) for 2 min 20 s, washed 4× with water and 1× with M9 buffer, and then suspended them in M9 buffer. We covered plates with a breathable seal (Excel Scientific AeraSeal, BS-25) and hatched eggs overnight at 20 °C.

Preparing plates with level agar

We prepared low-peptone NGM (2% w/v agar, 51 mM NaCl, 0.013% w/v bactopeptone in water) or full-peptone NGM (US Biological N1000, which uses 1.75% w/v agar, 51 mM NaCl, 0.25% w/v bactopeptone in water), microwaving to dissolve. After briefly cooling, we adjusted the solution to 13 µM cholesterol, 10 mM KH_2PO_4, 2.5 mM K_2HPO_4, 1 mM $CaCl_2$, and 1 mM $MgSO_4$ (note the lower-than-typical phosphate concentration). We pipetted 150 µL of NGM into 8-well strip plates (NEST 504201), either allowed it to set or placed the strips into our custom strip well holder, and then centrifuged at 1,000 × g for 10 min with an Eppendorf Centrifuge 5810 R. We then snapped the strips back into a 96-well frame. We inoculated each well with 1 µL of OP50 mixed with 0.025 mg/mL Nile red (MilliporeSigma, 72485) and 5 µM BODIPY 558/568 C12 (MilliporeSigma,

SML4163). After the OP50 dried, we used a P2 pipette to aspirate bleach-synchronized L1s and gently touched the tip on the agar to deposit a small number of animals. We wrapped plates in Parafilm and foil to keep moisture in and light out, and stored them in a 20 °C incubator between imaging sessions.

> Download **3D-printing files** for our custom 8-well strip centrifuge holder from NIH 3D.

Fabricating and testing the MeniSquish

We designed the MeniSquish and scraper using Fusion 360. We used Claude Opus 4.8 to design the custom strip-well holder. We sliced each model using Bambu Studio and printed all designs with a Bambu Lab X1E 3D printer. Xometry micro-machined the aluminum version. To make flat agar wells, we made NGM and then filled 96-well plates to just slightly overfull using a multichannel pipette. Overfilling helped prevent bubbles from forming when inserting the MeniSquish. We pushed the MeniSquish into the wells, allowing the agar to overflow. We allowed the agar to set for ~10 min before removing the MeniSquish.

Imaging and analysis

We imaged plates on a Nikon Ni-E upright widefield fluorescence microscope fitted with a Photometrics Kinetix sCMOS camera (6.5 µm pixel size). We used either a 2× objective, which captures a full well in a single frame, or a 4× objective with 2 × 2 tiling, and imaged once per day using brightfield and TRITC filter sets. We adjusted the brightness and contrast for all images with Fiji (v1.54p) [11].

For each multichannel brightfield/TRITC z-stack (11 planes, 2100 × 2100 px), we used the brightfield channel to identify the best-focused plane. To restrict the focus metric to the illuminated field of view and exclude well edges and vignetting artifacts, we applied a circular region of interest (ROI). We then downsampled each plane 4×, passed them through a Sobel edge filter, and computed the sum of edge magnitudes within the ROI per plane. We selected the plane with the highest relative sum as the in-focus frame, on the assumption that a sharply focused image yields the strongest high-frequency content (edges). We used the same focus index to extract the matching plane from the fluorescence (TRITC) channel.

Code, including our Opentrons protocols and in-focus plane slicer, is available in this GitHub repo.

AI usage

Chatting with Claude (Opus 4.8) sparked some ideas for modifications and approaches that we then tested. Claude suggested papers on relevant science, and we cited some of this literature after further reading.

We used Claude (Sonnet 4.6) to help write, clean up, comment, and review our code before we selectively incorporated its feedback.

We used Grammarly Enterprise to help copy-edit draft text to match Arcadia's style. We also used Claude (Sonnet 4.6 and Opus 4.8) to write text that we edited, suggest wording ideas, expand on summary text that we provided, copy-edit draft text to match Arcadia's style, clarify and streamline our text, suggest rearrangements to improve the pub's flow, and generate figure mockups.

Finally, we used Claude (Opus 4.7, 4.8, and 5.0) to provide scientific feedback on the pub and to check that feedback to verify that our conclusions were supported.

Key takeaways

Imaging small organisms like *C. elegans* in 96-well plates at scale is complicated by the curved surface (a meniscus) that forms when you pour liquid agar into each well. Animals occupy different depths and can't all be captured in one image. We found that centrifuging agar in 8-well strips and then snapping the flattened strips into a 96-well frame produces a flat imaging surface across all wells. The protocol uses only standard and 3D-printable lab equipment, scales smoothly from a few wells to a full plate, and is compatible with multichannel pipettes, liquid handlers, and automated microscopes. We also optimized the growth media by lowering phosphate and peptone concentrations, which eliminated salt crystal formation and thinned the bacterial lawn — both of which had interfered with image segmentation. The fluorescent food additives we tested, Nile red and BODIPY C12,

did not substantially help distinguish worms from background, and we're continuing to explore better options. We expect these approaches to be useful to anyone who cultures and images small organisms on agar in a high-throughput format.

Next steps

We're using this protocol as part of our ongoing efforts to track macroscopic traits in *C. elegans* across diverse genetic and chemical perturbations, and we plan to share updates as this work develops.

As noted above, the animals are difficult to segment against the OP50 field, and the dyes we tested (Nile red and BODIPY C12) didn't substantially improve the signal-to-noise ratio. We'd appreciate suggestions for dyes that might preferentially label *C. elegans* over OP50, accumulate in the animals over time, and transfer into eggs.

More broadly, we developed this protocol with agar-dwelling organisms in mind, and we're curious to see what other systems might benefit. If you use it with organisms other than *C. elegans*, we'd love to hear from you. Please share your feedback, questions, and results in the comments.

Contributors (A–Z)

- **Audrey Bell**: Visualization
- **Brae M. Bigge**: Editing, Experimental design, Supervision
- **Ben Braverman**: Methodology
- **Megan L. Hochstrasser**: Editing
- **Tyler Kennedy**: Conceptualization, Experimental design, Investigation, Methodology, Visualization, Writing
- **Ryan Lane**: Formal analysis, Software

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